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Transactivation of Human Immunodeficiency Virus Promoter by Human Herpesvirus 6

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Patients with acquired immunodeficiency syndrome (AIDS) are often infected with a number of other heterologous viruses in addition to the initial human immunodeficiency virus (HIV) infection, and these agents could act as potential reactivating agents of latent HIV. A new antigenically distinct herpesvirus, designated human herpesvirus 6 (HHV-6), has recently been isolated from patients with AIDS and has been shown to infect a number of different human cells, specifically human T cells, B cells, and glial cells. Since these are some of the same cells that harbor the AIDS virus, it is quite important to determine any interaction between this new herpesvirus and HIV. In this report, we demonstrate that HHV-6 can *trans*-activate the HIV promoter in human T-cell lines as measured by the expression of the bacterial gene chloramphenicol acetyltransferase. This indicates that stimulation of HIV gene expression by HHV-6 could play a role in HIV pathogenesis.

Infection of human helper T lymphocytes by human immunodeficiency virus (HIV) results in the development of acquired immunodeficiency syndrome (AIDS), which is characterized by the severe and persistent breakdown of the immune system (3, 4, 7). Human herpesviruses are responsible for a significant proportion of the collective morbidity and mortality reported in patients with AIDS. HIV replication has been shown only in a small proportion of T cells in vivo, and virus tends to persist for a prolonged period of time (3, 9, 20). The sequence of events needed to trigger the conversion of a persistent latent infection to an acute cytolytic HIV infection is not known. One of these events could be heterologous viral infections. Of potential major significance to our understanding of the relationship between HIV and heterologous viruses has been the in vitro demonstration of the activation of the HIV long terminal repeat (LTR), which contains the viral promoter, by other viruses. The early gene products of viruses such as herpes simplex virus type 1 (10), cytomegalovirus (2), and adenovirus (13) have been found to *trans*-activate the HIV LTR promoter activity. Human herpesvirus 6 (HHV-6) is a new herpesvirus, antigenically distinct from other human herpesviruses and has been isolated recently from the peripheral blood lymphocytes of patients with AIDS (8, 17), from patients with lymphoproliferative disorders (15), and from infants with exanthem subitum (19). It infects human T cells (including both T4+ and T8+ cells), B cells, and glioma cells in vitro. However, the target cell(s) for viral latency is not known. This study examines the in vitro interaction between HIV and HHV-6, and this preliminary report presents evidence for the *trans*-activation of the HIV promoter by HHV-6.

We used the prototype GS strain of HHV-6 (15). Two human T-cell lines, HSB-2 (ATCC-CCL 120.1. CCRF-HSB-2) and HuT-78 (ATCC-TIB 161), grown in RPMI 1640 with 10% serum supplement (CPSR-1; Sigma Chemical Co.), were used for the propagation of HHV-6. Infection of these cells with HHV-6 resulted in a characteristic aggregation and enlargement of infected cells by 3 to 6 days postinfection. Electron microscopic examination of thin sections of infected HSB-2 cells revealed large quantities of developing viral particles in the host cell nucleus as well as complete enveloped particles in cytoplasmic vacuoles and in the extracellular compartments (Fig. 1). These viruses exhibit a characteristic herpesvirus morphology (15), and the whole virus measured about 160 μm in diameter. More than 30 virus-specific peptides were detected in these infected cells (N. Balachandran, R. E. Amelse, W. W. Zhou, and B. R. Fegley, submitted for publication). To measure the infectivity, fresh HSB-2 cells (10^6) were infected with dilutions of either infected cell supernatant fluids or cell lysates. After 5 days, infected and uninfected cells were washed in phosphate-buffered saline, fixed on coverslides by cold acetone, and tested for the presence of viral antigens with an indirect immunofluorescence assay using a prestandardized dilution of a patient's serum (15). After 5 days postinfection, the culture supernates of HSB-2 cells usually yielded about 10^4 per ml of infective virus (a 50% tissue culture infective dose). The yields from HuT cells were usually about fivefold lower than that of HSB-2 cells.

The human T-cell line, HuT-78, was transfected with a plasmid that contained the HIV LTR region ligated upstream of the indicator chloramphenicol acetyltransferase (CAT) gene (Fig. 2). Construction of the HIV-CAT plasmid has been described previously (F. Kashanchi and C. Wood, Proc. Natl. Acad. Sci. USA, in press). The HIV LTR region of the plasmid contained both the 3′ untranslated region and the repeat sequences. HuT cells were transfected with the plasmid by protoplast fusion which is described in detail elsewhere (16). Briefly, bacterial cells that contained the plasmid were treated with lysozyme to form protoplasts and were then fused to HuT-78 cells using polyethylene glycol. After transfection, the cells were cocultured with HHV-6-infected HuT-78 or HSB-2 cells (4 days postinfection), culture supernatant fluid from these cells (5×10^4 per ml of infective virus [a 50% tissue culture infective dose]), or control uninfected cells. The T cells were collected 2 days later, and 2×10^6 cells were lysed by sonication. The cell extracts were assayed for CAT activity as described by Gorman et al. (5). [^{14}C]chloramphenicol (0.3 μCi) was incubated with 0.5 mM acetyl coenzyme A and 3 μl of cell extract in a final volume of 150 μl of 0.5 M Tris hydrochlo-

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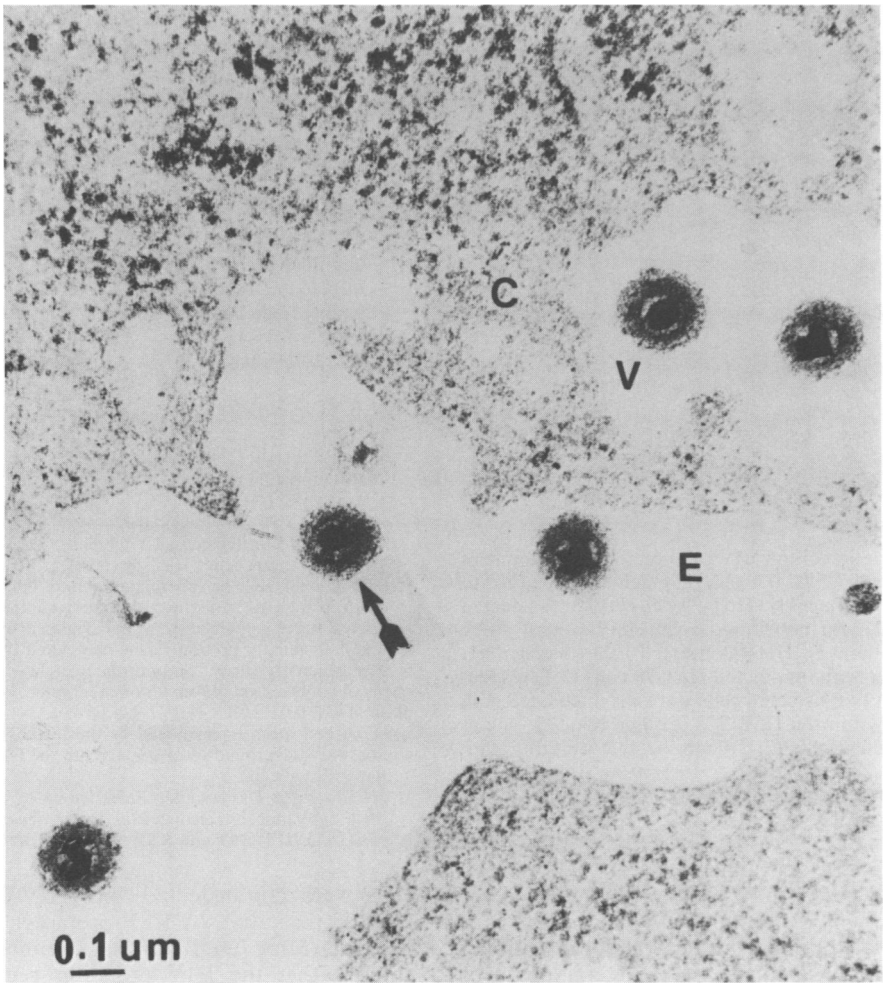

FIG. 1. Electron micrograph of HHV-6 infected HSB-2 cells on day 6. Thin sections of glutaraldehyde-fixed infected cells were made and stained with uranyl acetate and lead citrate. Grids were examined under a JEOL-100 CX transmission electron microscope. Enveloped intracytoplasmic (C) viral particles in vacuoles (V) and extracellular (E) viral particles are shown. Arrow indicates the projections on the viral envelope.

ride (pH 7.4) for 3 h at 37°C. The radioactive chloramphenicol was then extracted with ethyl acetate and analyzed by thin-layer chromatography in chloroform-methanol (95:5). The percentage of chloramphenicol converted to acetylated forms was determined for each sample.

The results of the HIV-LTR-directed CAT gene expression with or without the addition of HHV-6 are shown in Fig. 3 and Table 1. Little CAT activity was detected in T cells transfected with HIV-CAT alone (Fig. 3, lane 2). When the transfected cells were cocultured with uninfected HuT-78 or HSB-2 cells, there was no significant change in CAT enzymatic activity (Fig. 3, lanes 5 and 8). However, when these T cells were infected with culture supernatant fluids containing HHV-6 (Fig. 3, lanes 3 and 6) or cocultured with HHV-6-infected HuT-78 or HSB-2 cells (Fig. 3, lanes 4 and 7), CAT expression was dramatically increased (42- to 66-fold). These data indicate that infection of HIV-CAT-transfected T cells with HHV-6 *trans*-activates the HIV-LTR-directed gene expression. Since our HIV-CAT construct contains the simian virus 40 enhancer (Fig. 2), it is possible that HHV-6 activates the simian virus 40 enhancer rather than the HIV promoter itself. A control plasmid that

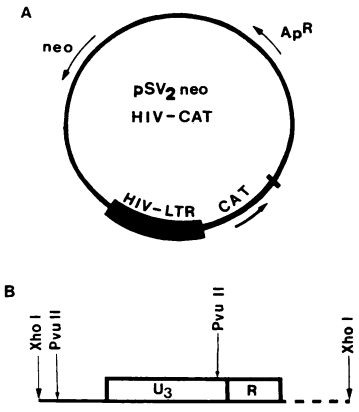

FIG. 2. (A) Schematic diagram of the HIV-CAT construct. The HIV-LTR was ligated to the CAT gene and inserted into the pSV$_2$neo plasmid. (B) The LTR insert was the *Xho*I fragment that contained the untranslated (U$_3$) and repeat sequence (R) regions as well as a portion of the vector sequence (- - -).

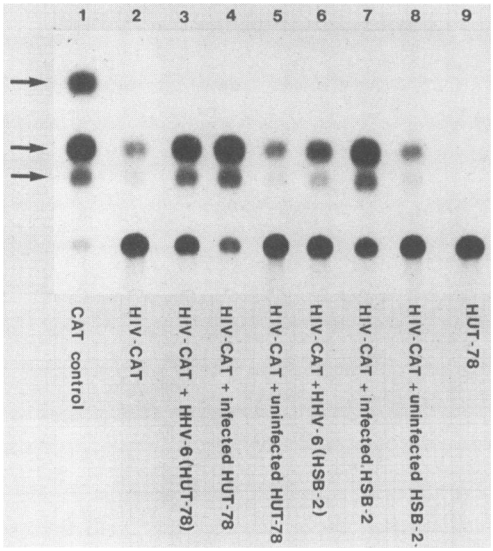

FIG. 3. HHV-6 activation of the HIV promoter. A 1-U sample of CAT enzyme converted 97% of the chloramphenicol substrate to acetylated forms (arrows) (lane 1). HuT-78 cells were transfected with HIV-CAT (lane 2) and cocultured with HHV-6 viral stock grown in HuT-78 cells (lane 3), HHV-6-infected HuT-78 cells (2:1, respectively) (lane 4), or with uninfected HuT-78 cells (2:1, respectively) (lane 5). HIV-CAT-transfected cells were also incubated with HHV-6 viral stock grown in the HSB-2 T-cell line (lane 6), cocultured with HSB-2 infected cells (2:1) (lane 7), or cocultured with uninfected HSB-2 cells (2:1) (lane 8). Lysates from untransfected HuT-78 did not contain detectable CAT activity (lane 9).

TABLE 1. *trans*-Activation of HIV LTR CAT after infection with HHV-6

Plasmid(s) + T cells (ratio[a])[b]	% CAT activity[c]	Fold activation[d]
HIV-CAT		
+ None	1.0 ± 0.4	
+ Uninfected (10:1)	0.7 ± 0.2	
+ Infected (10:1)	23.3 ± 2.4	33
+ Uninfected (5:1)	1.3 ± 0.4	
+ Infected (5:1)	59.0 ± 6	45
+ Uninfected (2:1)	1.0 ± 0.3	
+ Infected (2:1)	66.0 ± 5	66
+ Uninfected (1:1)	1.4 ± 0.3	
+ Infected (1:1)	83.0 ± 6	59
+ 5×10^4 $TCID_{50}$[e]	52.0 ± 9	52
HIV-CAT + RSV-tat		
+ None	24.0 ± 2	24
+ Uninfected (2:1)	19.5 ± 4	20
+ Infected (2:1)	75.0 ± 5	3.6[f]
+ 5×10^4 $TCID_{50}$	45.0 ± 4	2[f]

[a] Number of transfected cells: number of HHV-6-infected HuT-78 cells.
[b] HIV-CAT-transfected HuT-78 cells were cocultured in the presence of HHV-6-infected cells or supernatant culture fluid that contained HHV-6.
[c] CAT enzymatic activities were derived from data obtained from 3-h reactions. Percent conversion was calculated by dividing the radioactivity (counts per minute) present in the acetylated chloramphenicol spot by the total radioactivity present in both the acetylated and unacetylated chloramphenicol spots on thin-layer chromatography. Percent CAT activity for no plasmid was 0.05 ± 0.
[d] Fold activation is calculated as the increase in CAT activity over the appropriate background levels, which are for HIV-CAT alone or HIV-CAT with uninfected cells.
[e] $TCID_{50}$, 50% Tissue culture infective dose.
[f] Fold activation is calculated as the increase in CAT activity over the activity from HIV-CAT and RSV-tat with or without uninfected HuT cells.

lacked the simian virus 40 enhancer also showed similar *trans*-activating activity (66- and 71-fold) in the presence of HHV-6 (data not shown), confirming the observation that HHV-6 acts on the HIV promoter rather than on the simian virus 40 enhancer. Table 1 summarizes the average percent acetylation of chloramphenicol from several experiments. This *trans*-activating effect seems to be dependent on the amount of HHV-6 used to infect the transiently transfected T cells. Approximately 31% of HuT cells and 27% of HSB-2 cells expressed viral antigens as measured by an indirect immunofluorescence assay (data not shown). When transfected cells are cocultured with fewer virus-infected cells (10:1) the amount of *trans*-activation is lower (33×) than that in a cocultivation with an equal number of transfected and infected cells (1:1, 59×), demonstrating that this effect is dose dependent. The CAT activity of T cells that were cocultured with HHV-6 infected cells was consistently higher than that of T cells that were infected directly with virus. With other herpesviruses, viral envelope glycoproteins are expressed on the infected cell membrane and mediate cell-to-cell fusion (14). Such a phenomonon has also been demonstrated with HHV-6 (1, 15), and the fusion of infected cells with transfected T cells results in the delivery of a higher quantity of infective intracellular virus, presynthesized HHV-6 viral proteins, or both. This could explain the greater *trans*-activation seen with HHV-6-infected cells versus HHV-6 alone.

HIV has an endogenous *trans*-activator protein called tat. This *trans*-activator has been characterized as a 14-kilodalton protein that interacts directly or indirectly via cellular factors with the LTR of HIV to increase the steady-state level of viral mRNA (12, 18). To determine whether HHV-6 infection has any effect on tat *trans*-activation, the HuT-78 cells were cotransfected with HIV-CAT and RSV-tat, a plasmid that used the RSV promoter region to express the *tat* gene (Mira Jung, personal communication). The results showed that the HIV promoter region was consistently responsive to *trans*-activation with tat by increasing CAT activity at least 24-fold (Table 1). Although other reports have shown higher *trans*-activation of the HIV-LTR, 24-fold activation was consistently observed in these T cells, possibly because of the differences in transfection procedures and tat constructs that were used. Cells transfected with HIV-CAT and RSV-tat which were then infected with HHV-6 showed a further 3.6-fold stimulation above that of cells induced by tat alone (Table 1). However, when compared with the results of previous experiments using the HIV-CAT plasmid alone and then infection with HHV-6, the increase in the activation of the HIV promoter is only slight. This may be due to experimental variation or may reflect the lack of additional activation by tat in the presence of HHV-6. These results nevertheless suggest that HHV-6 does not compete with tat in order to *trans*-activate the HIV promoter but will increase promoter activity even when tat is present. However, it cannot be determined whether there is an additive or cooperative effect between tat and HHV-6 on the HIV promoter.

The results presented here are similar to those of other studies that have demonstrated the *trans*-activation of HIV-LTR by heterologous viruses (2, 10, 13). These studies have shown that several immediate-early genes of herpes simplex virus-1 (10), and human cytomegalovirus (2), as well as early products from adenovirus (13), will *trans*-activate the HIV promoter. This suggests that infection by HHV-6 or other viruses may be playing an important role in converting a

latent HIV infection to an acute phase. The molecular events that lead to this conversion may simply be the *trans*-activation of the HIV promoter, which in turn stimulates HIV viral replication. This preliminary study does not identify the HHV-6 viral products responsible for inducing the *trans*-activation of HIV; however, from the previous studies it would likely involve early gene expression. The DNA of HHV-6 is estimated to be more than 150 kilobases (15), and further studies are under way to identify the HHV-6 genes and characterize the proteins involved or required for the *trans*-activation of the HIV promoter.

The mechanisms involved in the *trans*-activation of HIV-LTR by other viruses is not fully understood but may involve direct interactions of viral gene products with HIV promoter or indirectly via cellular intermediates. A number of cellular factors have been identified and have been shown to bind to defined sequences of the HIV promoter region (6, 11). It is not known whether any of these factors can be stimulated by HHV-6 infection. Examination of various deletion mutants of the HIV-LTR region would also help to determine which sequences are necessary for *trans*-activation by HHV-6. A comparison of the HHV-6 target sequences on the HIV-LTR promoter with the HIV sequences known to be required by other viruses and cellular factors would allow for the examination of common features required by several viruses for *trans*-activation. If these sequences are also known to be regions in which cellular factors, such as Sp1 and NF-kB, will bind, it may indicate a cooperation between viral proteins and cellular factors.

HHV-6 has been isolated from infants, which suggests that infections occur early in life (19). Isolation from patients with AIDS could merely be due to reactivation of latent HHV-6 under immunosuppressed conditions. However, the similarity of target cells for HHV-6 to those for HIV and our present data suggest that HHV-6 may play a role in the pathogenesis of HIV infection and AIDS. Moreover, HHV-6 is extremely cytolytic for human T cells in vitro (1, 8, 15, 19), and reactivation in individuals with HIV infection could also contribute to the depletion of T cells. Further work is in progress to determine any possible interactions between these two viruses.

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